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Monoclonal antibodies against chordin

Use in structural and immunohistochemical studies

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Eight MAbs have been developed against chordin and designated as At2–At9. It is shown that all antibodies are directed against identical, spatially overlapping or closely positioned epitopes of chordin. The chordin molecule has repetitive sites wherein epitopes for the eight MAbs are located. This site lies within a proteinase-resistant fragment of chordin, presumably a glycopeptide, of molecular mass between 2 and 10 kDa. Fluorescence staining of cryostat sections from stellate sturgeon with the use of At5 (indirect Coons' method) has revealed a positive reaction with notochord cells and sheath and with the spinal cord. No significant reaction with cartilage, muscle and kidney was detected.

Chordin; Notochord; Repetitive epitope; Restricted tissue specifity; (Nervous tissue)

1. INTRODUCTION

Chordin is a characteristic component of notochord of sturgeoneous fishes [1–3]. It is a protein of M_r about 100000 as judged from gel filtration data and with p*I* close to 1. Chordin displays polydispersity as revealed by SDS-PAGE and other methods [3,4]. It has been suggested that the chordin molecule contains carbohydrate chains of different lengths [4]. The antigenicity of chordin is affected by treatment with proteinase [1]. During giant sturgeon (*Huso huso*) embryonic development chordin can first be detected in the embryos by RIA at stage 32 [3,4] (tip of the tail touching the head [5]); after hatching the chordin content increases rapidly [3,4]. Chordin-specific epitopes have been found by RIA in human and rabbit intervertebral discs, human and rabbit brain and in a tissue sample of human rectal adenocarcinoma [6,7]. Here we present the first structural and immunohistochemical data obtained with the use of MAbs developed against chordin.

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2. MATERIALS AND METHODS

MAbs against chordin were produced according to Köhler and Milstein [8] and the procedure has been described in detail [9]. BALB/c mice were immunized by repetitive intraperitoneal injections of notochord homogenate [1]. Screening of hybridoma supernatants was performed by solid-phase RIA with the use of purified chordin [10]. MAbs were isolated from ascitic fluid through ammonium sulfate precipitation and DEAE-cellulose chromatography [11]. Judging from SDS-PAGE MAb At5 was of the IgG class and MAbs At2–At4 and At7–At9 of IgM. Liquid-phase RIA for chordin has been described previously [3,4]. The direction of MAbs to epitopes of chordin was studied by the co-titration method [12] with the use of solid-phase RIA [10]. Student's *t*-test was employed for statistical treatment of the data. The

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true significance of the determined values lies within the limits shown in the figures with a probability of 95%.

Ouchterlony double immunodiffusion [13] was performed in 1.5% agarose gel made in PBS at 4°C for 20 h. Preparation of the *H. huso* notochord cell extract was according to [1]. The absorbence of the extract measured at 280 nm in a 1 cm cell was 0.75. Incubation of the notochord extract with papain (0.25 mg/ml) was performed at 60°C for 20 h in the presence of cysteine and EDTA as described in [1]. After incubation the papain was inactivated with iodoacetamide. Incubation with pronase (1 mg/ml) was performed at 37°C for 2 days. Another portion of the enzyme was then added to the same concentration and incubation allowed to proceed for another 3 days. At the start of incubation the sample surface in the test-tube was covered with a 2 mm layer of toluene. Extraction of the pronase hydrolysate was carried out once with a phenol-chloroform mixture as in [14] and twice with chloroform. Gel chromatography through a column of TSK gel Toyopearl HW-40F (Toyo Soda), 1.1 × 55 cm) was performed at a flow rate of 9.6 ml/h. The column was equilibrated with 0.4 M NaCl and 0.01 M Na phosphate (pH 7.4). Fractions of 1.3 ml were collected.

An *Acipenser stellatus* for immunohistochemical studies (body length 15 cm) was a generous gift from Dr Yu.N. Sbikin. The cryostat sections of pieces with notochord and adjacent tissues were cut 5 μm thick. The sections were fixed with 96% ethanol, acetone or 4% formaldehyde and processed with antibodies according to Coons' indirect method [15]. MAb At5 was introduced at a concentration of 0.05 mg/ml. Pooled MAbs against human type I and III collagens (a generous gift from Dr G.L. Idelson) served as a control. The immunohistologic preparations were observed and photographed with a fluorescence microscope (Ortoplan, Leitz FR6).

Papain was from Loba-Chemie, pronase from Calbiochem, and the FITC conjugate of rabbit anti-mouse antibodies from Sigma.

3. RESULTS AND DISCUSSION

Eight hybridoma clones producing chordin-specific antibodies were produced and independently selected in this study. The corresponding MAbs were designated at At2–At9. To determine whether the MAbs react with the same or different epitopes of the chordin molecule, experiments were performed in which pairs of antibodies were co-titrated and the same antibodies were titrated separately. A typical example of such an experiment is given in fig.1. It can be seen from the figure that each of the three titration curves reaches a plateau with an increase in antibody concentration. The saturation binding level for the two co-titrated antibodies does not exceed that of each individual antibody. Hence, it is obvious that these two antibodies compete for an epitope of

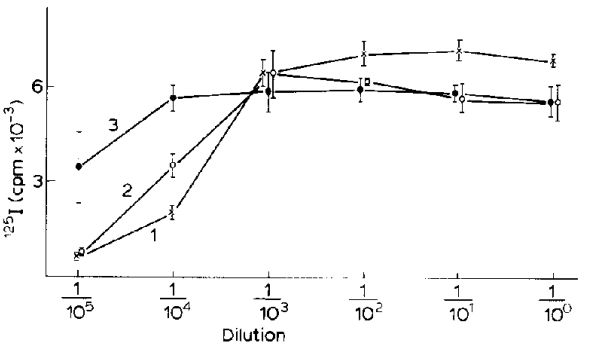

Fig.1. Solid-phase RIA. Binding of ^{125}I-rabbit anti-mouse antibodies in the presence of At4 (1), At7 (2) and both antibodies with the same partial concentrations (3). Dilution = 1:(100 × 5^n).

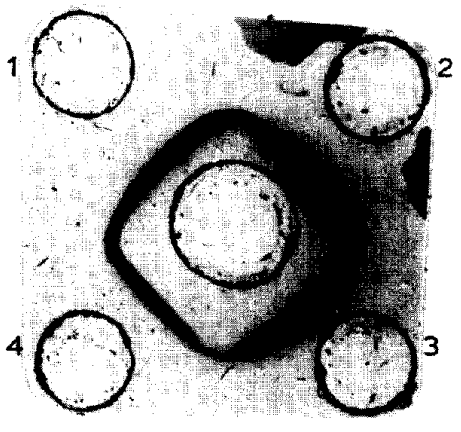

Fig.2. Double diffusion Ouchterlony test. Inner well, chordin, 5 μg/ml; outer wells, At6 at 500 μg/ml (1), 250 μg/ml (2), 125 μg/ml (3), 62.5 μg/ml (4).

chordin. Similar experiments were performed with other pairs of antibodies (At2-At9, At2-At4, At3-At4, At3-At7, At3-At8, At5-At6, At6-At9, At7-At9, At8-At9) and in all cases competition of MAbs was observed. On the grounds of the co-titration experiments we conclude that the eight MAbs tested were directed to identical, spatially overlapping or closely positioned epitopes of chordin. We suggest that the epitopes recognized by MAbs At2–At9 be designated as P2–P9, respectively. That eight independently selected hybridomas produce MAbs to the same site of the chordin molecule indicates that this site is highly immunogenic for mice.

The method of double immunodiffusion in agarose gel was used to determine whether the site of the chordin molecule which is recognized by MAbs At2–At9 is repetitive. It is known that in the case of non-repetitive epitopes MAbs do not form immunoprecipitates with antigens. If an epitope is present in two copies per molecule of the antigen the formation of an immunoprecipitate with an MAb can be observed. In such cases polyethylene glycol of 6–8 kDa is necessary as a stabilizing agent [16–18]. The precipitates sometimes disappear from the gel with washing [16]. In our case each of the eight MAbs formed a stable immunoprecipitate with chordin in double immunodiffusion experiments. An example of a Coomassie-stained precipitate of chordin with MAb At6 is given in fig.2. If an MAb of non-chordin specificity or mouse normal serum was taken in an experiment, no precipitation lines in the gel were observed (not shown). Double diffusion experiments with MAbs

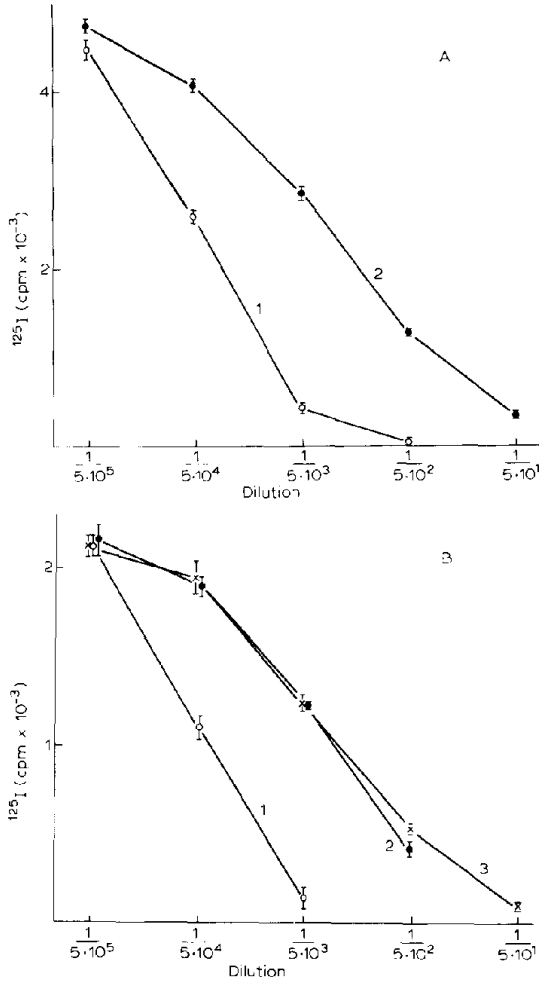

Fig.3. Liquid-phase competitive-binding RIA. Titration of *H. huso* notochord extract treated with papain (A) and pronase (B). (1) Inhibition in control (freshly thawed aliquots), (2) inhibition by papain or pronase hydrolysate, (3) inhibition by pronase hydrolysate extracted with phenol and chloroform. Dilution = 1:10^n.

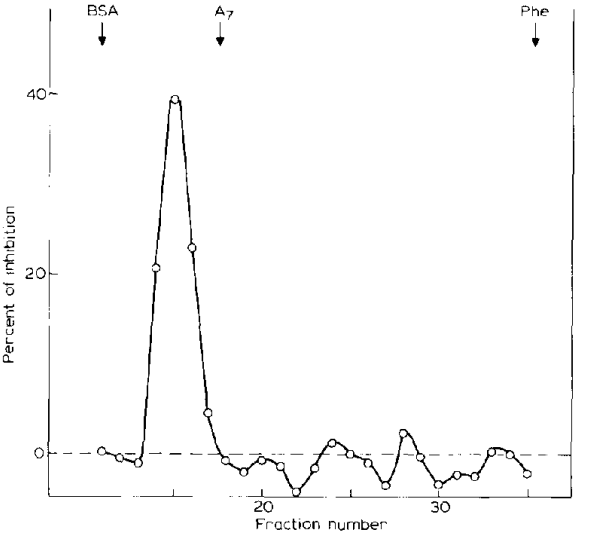

Fig.4. Liquid-phase RIA. Inhibition of ^{125}I-chordin binding to At8 by fraction material from the HW-40 column. Percent of inhibition = 100 − (cpm in the probe):(cpm in the control) × 100. Binding in the control is 32.051 cpm. Mean values of three parallel determinations are used for each point. Arrows indicate positions of bovine serum albumin (BSA), heptariboadenylate (A$_7$) and phenylalanine (Phe).

show that the site of the chordin molecule within which epitopes of the P family are located is repetitive.

In a previous paper the sensitivity of chordin to digestion by papain and trypsin has been demonstrated [1]. It was not clear, however, whether chordin epitopes might be completely destroyed by proteolysis. We attempted to perform exhaustive proteolysis by prolonged incubations of the notochord extract with papain and pronase (see section 2). In both cases chordin completely lost the ability to form immunoprecipitation peaks in rocket immunoelectrophoresis (not shown). Proteinase hydrolysates were also tested in

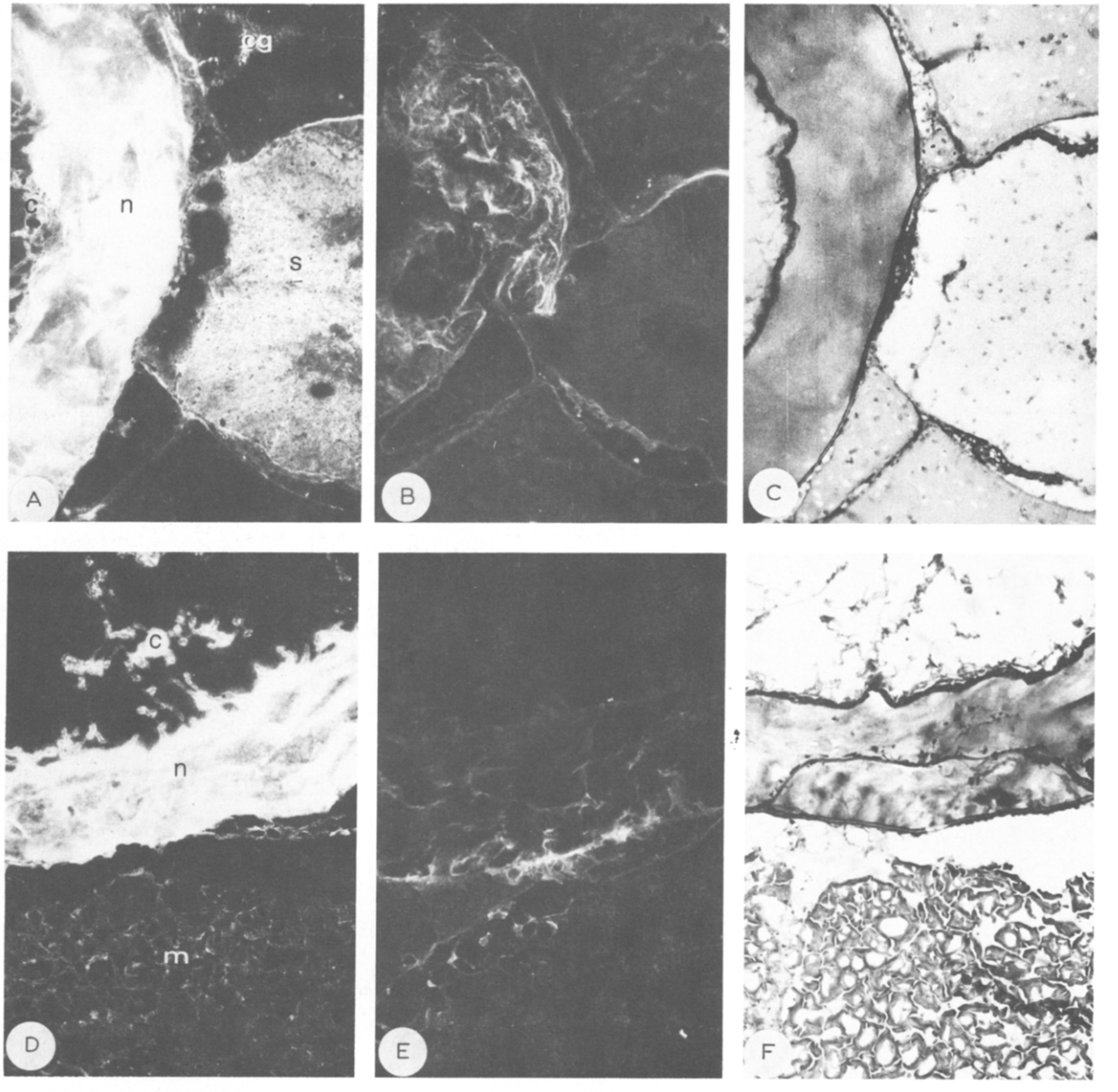

Fig.5. Immunohistochemical study of chordin P5 epitope localization. (A–F) Serial cryostat sections (× 85). (A,D) Specific fluorescence of notochord sheath (n), notochord cells (c), spinal cord (s). (m) Muscle, (cg) cartilage. (B,E) Controls (incubation with pooled MAbs to type I and III collagens). (C,F) Hematoxylin-eosin staining.

competitive-binding liquid-phase RIA for the ability to inhibit binding of chordin to MAb At8 (fig.3). In the case of papain the enzyme activity was blocked at the end of incubation by addition of iodoacetamide (if autolysis was not completed); in the case of pronase no inhibition of the enzyme was applied, but the deproteinized pronase hydrolysate was assayed in parallel (curve 3). One can see that after papain and pronase treatments the inhibitory capacities of both hydrolysates decreased, but a 90–95% inhibition of the chordin-At8 complex formation can be achieved. The same value of inhibition was achieved for the chordin-At2 complex (not shown).

Chromatography on TSK gel HW-40 was performed to evaluate the dimensions of the pronase-resistant antigenic fragment(s) of chordin. It can be seen from fig.4. that a homogeneous fragment which reacts with At8 elutes between V_0 and V_e for heptariboadenylate (2.4 kDa). According to the Toyo Soda manual, 10 kDa proteins and 7 kDa dextrans are excluded from the HW-40 gel. Thus, the molecular mass of the proteinase-resistant fragment of chordin should be between 2 and 10 kDa. We assume that these repetitive homogeneous proteinase-resistant hydrophilic fragments of chordin are glycopeptides.

The immunohistochemical localization of a chordin-specific epitope was studied in the next series of experiments. Cryostat tissue sections of *A. stellatus* (stellate sturgeon) were assayed with MAb At5 (fig.5). Intense fluorescence was observed with notochord sheath and notochord cells. It should be pointed out that gigantic vacuolyzed cells of notochord did not endure cryostat sectioning well, thus only single cells and their remains could be seen. Moderate fluorescence was observed with the spinal cord. Very weak fluorescence was observed among the muscular fibers on transverse sections, but control sections often showed the same picture. Cartilage plates around the spinal cord were completely dark, like kidney tissue (not shown). Different fixatives did not affect the distribution of structures positively stained with At5.

In a previous paper we reported that chordin was not detected in a brain extract of giant sturgeon (in those studies rocket-line immunoelectrophoresis was used) [1]. Later, a more sensitive RIA method was used to show that some component(s) of human and rabbit brain extracts significantly cross-react with polyclonal rabbit antibodies to chordin [7]. Since the components cross-reacting with antichordin antibodies were not found in more than three dozen extracts from various human and rabbit tissues, chordin was defined as an antigen with a restricted tissue specificity [7]. The detection of chordin-specific epitopes by the immunohistochemical method in notochordal and nervous tissues is in good agreement with the results of our previous investigations. The observation that a common chordin-specific epitope is present in both tissues may be useful in future studies of embryonic tissue differentiation.

4. CONCLUSIONS

(i) The chordin molecule contains a repetitive site which is highly immunogenic for mice.
(ii) The antigenicity of chordin decreases after exhaustive proteolysis with papain and pronase. A proteinase-resistant fragment of chordin of between 2 and 10 kDa has been identified by reaction with MAbs.
(iii) Immunofluorescence studies have revealed the reaction of a chordin-specific MAb with notochord cells and sheath and with spinal cord of stellate sturgeon. Cartilage, kidney and muscle were negative in the immunohistochemical reaction.

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